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RNA silencing in viral infections: insights from poliovirus

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Abstract

RNA interference or RNA silencing is a dsRNA guided mechanism that mediates sequence specific degradation of RNA. The recent demonstration that RNA interference can be used to inhibit virus replication has initiated an exciting field of research: first, as a potential novel antiviral therapeutic approach and, second, as a tool for dissecting virus–host interactions. Here we review and discuss the current data and perspectives on the use of RNA interference in the study of poliovirus as a model for positive strand RNA viruses.

Keywords: RNA interference; RNA silencing; siRNA; Poliovirus; Picornaviridae

1. Introduction

Clearance of viral pathogens from a variety of hosts is often the result of the destruction of infected cells. However, mechanisms that enable cell survival following infection have been described (Guidotti and Chisari, 2001). Studies in plants suggest that one of such mechanisms may be based on RNA-induced gene silencing, now referred to as RNA silencing or RNA interference (RNAi). This ancient pathway is conserved among various species from different kingdoms (fungi, animals and plants) and it seems to control gene expression at transcriptional and post-transcriptional levels (Bernstein et al., 2001b; Hammond et al., 2001b).

The post-transcriptional activity of the RNAi machinery to degrade cytoplasmic RNA in a sequence specific manner is key to its antiviral function. Biochemical studies indicate that RNAi proceeds via a two-step mechanism. In the first step, long dsRNAs are produced locally or taken up by the cells and cleaved by the RNase III-like nuclease Dicer (Bernstein et al., 2001a), which generates 21–23 nucleotide duplex RNAs, called small interfering RNAs (siRNA). In the second step, siRNAs are incorporated into a multicomponent nuclease complex, the RNA-induced silencing complex (RISC) (Hammond et al., 2001a). The antisense strand of the duplex serves as a guide that directs RISC to recognize and cleave cognate mRNAs (Martinez et al., 2002a) (Fig. 1b). Because of the extreme specificity and efficiency of the RNAi machinery, this mechanism has the ability to clear plant and mammalian cells from viral infection (Gitlin and Andino, 2003; Randall et al., 2003; Waterhouse et al., 2001).

2. Multiple functions of RNAi

The RNAi process is very efficient: a few dsRNA molecules can trigger inactivation of a continuously transcribed target mRNA for prolonged periods of time (Ruvkun, 2001). The RNAi-induced inactivation persists through cell division and in some organisms can spread to untreated cells and tissues; when the RNAi spreads into germ line cells it can even be inherited by subsequent generations (Hammond et al., 2001b). Although amplification and spreading of RNA silencing have been demonstrated in plants, nematodes and in vitro in *Drosophila* extracts (Fire et al., 1998; Lipardi et al., 2001; Lucas et al., 2001; Roignant et al., 2003) they have not been observed in mammalian systems (Chi et al., 2003; Stein et al., 2003).

A role for RNAi pathways in the regulation of endogenous genes was suggested through the analysis of plants and animals containing dysfunctional RNAi components. For example, mutations in a component of RISC cause pleiotropic developmental abnormalities in *Arabidopsis* (Bohmert et al., 1998). A mutation in the *Arabidopsis* Dicer orthologue causes defects in leaf development and overproliferation of floral meristems (Jacobsen et al., 1999). Mutations in Argonaute family members in *Drosophila* impact normal development with drastic effects in neuronal devel-

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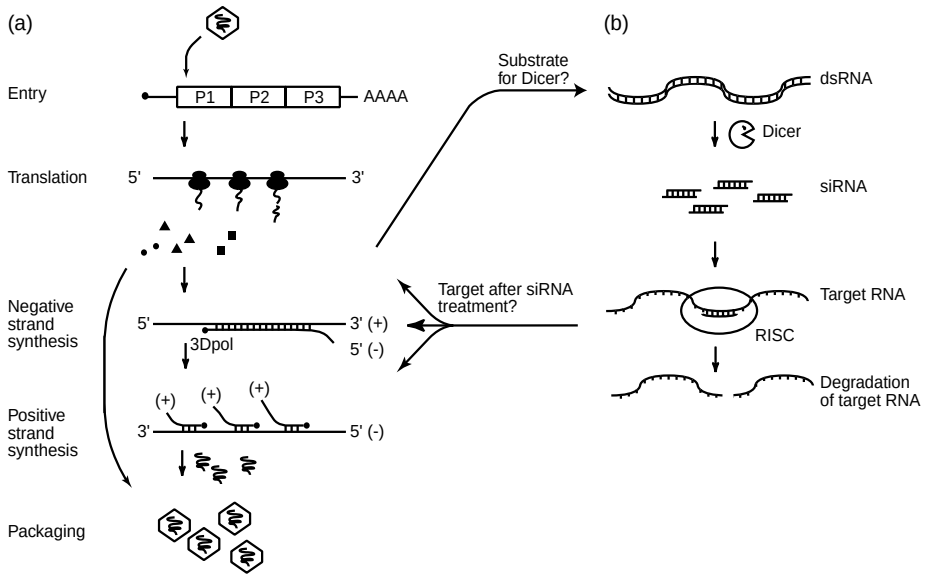

Fig. 1. (a) Overview of poliovirus life cycle. After receptor mediated entry and uncoating of the virus, the viral RNA is translated in a cap-independent fashion by the host cell machinery. The viral RNA is used as a template for negative strand synthesis by the virus encoded 3D polymerase. The negative strand is subsequently used as a template for the production of positive viral RNA strands, which are packaged into new virions. (b) Overview of RNAi pathway. The central component is dsRNA which is converted by Dicer into siRNA, small dsRNA of ~21 nucleotides length with a two nucleotide overhang at the 3′ end. One strand is incorporated in the RISC complex and guides recognition and cleavage of the target RNA. Open questions with respect to RNA silencing of positive strand RNA viruses include whether dsRNA intermediates produced during virus replication are a target for the RISC complex, whether viruses encode proteins that inhibit the RNAi pathway and which of the RNA strands are best targeted after introduction of synthetic siRNAs.

opment (Kataoka et al., 2001), and mutations in another RISC component, piwi, result in defects in both germline stem-cell proliferation and maintenance (Cox et al., 1998).

The emerging view is that RNA silencing is part of a sophisticated network of interconnected pathways for cellular defense (pathogen resistance and stabilization of mobile genetic elements), RNA surveillance (chromatin remodeling, genome organization and stability) and development. In addition, RNAi may become a powerful tool to manipulate gene expression experimentally. Here we review and discuss the current data and perspectives on the use of RNAi in the study of positive strand RNA viruses. Indeed RNAi opens up exciting possibilities for virus research: first, as a potential novel antiviral therapeutic approach and, second, as a tool for dissecting virus–host interactions.

3. Antiviral function of RNAi

There is a good deal of genetic support for the importance of RNAi in genome defense. In plants, RNAi is clearly involved in the response to viruses: *Arabidopsis* strains defective in post transcriptional gene silencing are more susceptible to virus infections (Mourrain et al., 2000), and a substantial number of plant viruses encode proteins that counter silencing (Brigneti et al., 1998; Voinnet, 2001; Voinnet et al., 1999). RNA silencing also appears to contribute to antiviral defense in invertebrates as it was shown by studies of flock house virus (FHV) and its interaction with the RNA silencing machinery in *Drosophila* cells (Li et al., 2002). Furthermore, many *C. elegans* mutants deficient in RNAi show transposon mobilization with obvious implications for genome stability (Ketting et al., 1999). A key question that remains unanswered is whether RNAi has a role as a natural antiviral defense in mammals. Five critical issues have recently been proposed to establish whether RNAi plays a role in natural defense against viruses (Gitlin and Andino, 2003):

(1) Generation of siRNA during the course of a natural infection.
(2) Up-regulation of RNAi machinery components during viral infection.
(3) Viral mechanisms to suppress or escape RNAi.
(4) Increased susceptibility to viral infection after mutation or deletion of RNAi components.
(5) Local or systemic spread of an RNAi-based antiviral response.

Even though synthetic siRNA transfected into mammalian cells can inhibit positive strand virus replication (Gitlin and Andino, 2003; Kapadia et al., 2003; Randall et al., 2003; Seo et al., 2003; Wilson et al., 2003), it is not clear whether RNA silencing can be elicited naturally during these viral infections. Although the replication intermediates may contain long stretches of dsRNA, especially during negative strand synthesis, it is not know whether they can be targeted for processing by Dicer. Localization of dsRNA intermediates in membranous and/or proteinous replication complexes may

shield these intermediates from the RNAi machinery. Answers to these and other related issues have not been obtained yet, but an exciting field of research has been opened up.

4. Poliovirus as a model system to study RNAi

Until the 1950s, poliovirus (PV), the etiologic agent of poliomyelitis, crippled thousands of children every year. Poliovirus is able to invade the nervous system, and can cause total paralysis in a matter of hours. The virus enters the body through the mouth and multiplies in the tonsils and the Peyer's patches of the small intestine before spreading to other sites by a viraemia. Initial symptoms are fever, fatigue, headache, vomiting, and stiffness in the neck and pain in the limbs. One in 200 infections leads to irreversible paralysis (usually in the legs). Among those paralyzed, 5–10% die when their breathing muscles become immobilized (Minor, 1997).

After the introduction of effective vaccines in the late 1950s (inactivated polio vaccine (IPV)) and early 1960s (oral polio vaccine (OPV)), poliomyelitis was brought under control. More recently, thanks to a global effort to eradicate poliovirus, the disease has been eliminated from most of the world, and only seven countries worldwide remain polio-endemic (http://www.polioeradication.org). However, as we face the final stages of the Poliovirus Global Eradication Initiative it is important to develop effective anti-poliovirus therapies.

Poliovirus has served as an excellent model to study the molecular and cellular events that lead to viral replication. As a consequence, much is known about its biology, life cycle and interactions with the host. Poliovirus is a positive-stranded RNA virus, member of the family Picornaviridae. The genome comprises a long open reading frame encoding a single polyprotein of more than 2000 amino acids, which is processed into functional proteins by virally encoded proteases. The poliovirus polyprotein can be divided into three major parts (P1–P3). The P1 region contains the four structural proteins (VP1–VP4) that constitute the capsid. Proteins derived from the P2 and the P3 region are involved in viral replication. After receptor-mediated entry, the viral genome is directly translated by the cellular translation machinery. The same viral RNA is then used as

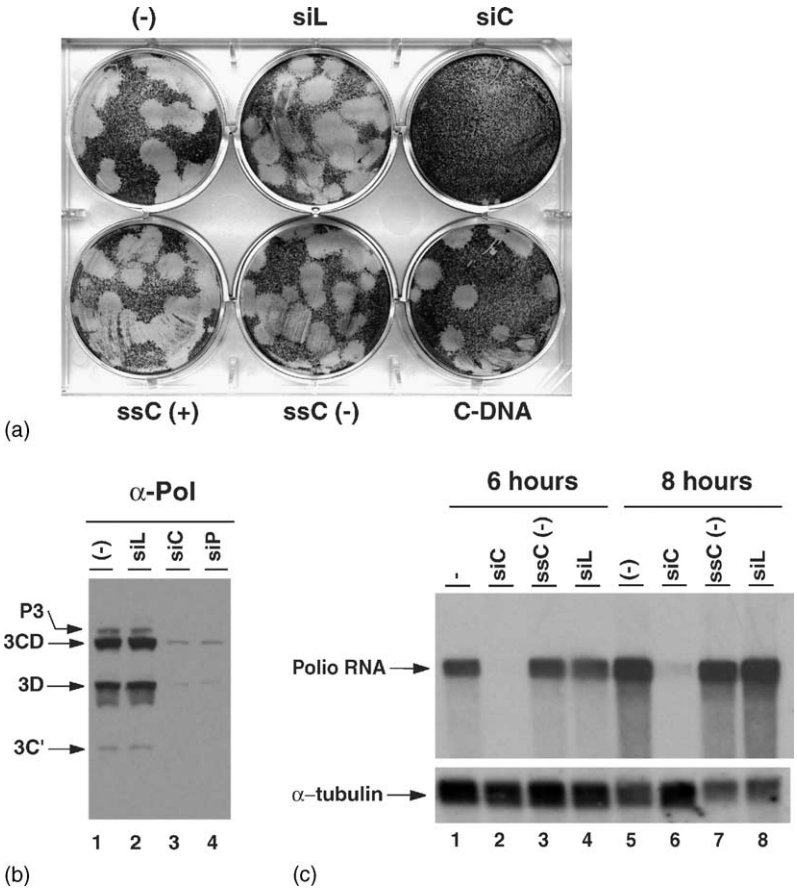

Fig. 2. Poliovirus replication can be suppressed by siRNA. (a) Pretreatment of HeLa cells with siRNA directed against the capsid region (siC) is able to prevent plaque formation. Suppression of replication is not observed after treatment of cells with single stranded RNA (ssC(+) and ssC(−)), an unrelated siRNA (directed against luciferase (siL)), or dsDNA of identical sequence of siC (C-DNA). (b) Western blot analysis with anti $3D^{pol}$ antibody of poliovirus infected cells. siP indicates siRNA directed against the poliovirus polymerase region. (c) Northern blot analysis of poliovirus infected cells at 6 and 8 h after infection. Reprinted from Gitlin et al. (2002) with permission.

template for negative strand RNA synthesis that results in the formation of a double-stranded RNA replicative form (RF). The negative strand RNA can in turn serve as a template for synthesis of new positive strand RNA. Replication occurs in replication complexes tightly associated with smooth membrane vesicles, which accumulate in the cytoplasm during viral infection (Fig. 1a) (Rueckert, 1996).

Can RNAi suppress replication of this highly lytic virus? In a recent study, Gitlin et al. (2002) used siRNA directed against the poliovirus genome to show that RNAi can effectively prevent viral replication in mammalian cells (Fig. 2). Pretreatment of human and murine cells with double-stranded siRNA to the poliovirus genome (capsid and 3D polymerase) prevented plaque formation and resulted in a 100-fold reduction of the progeny titer in one-step growth curves. In these experiments, siRNAs promote clearance of the virus from most infected cells. The RNAi antiviral effect, for which the mechanistic detail remains to be elucidated, is sequence-specific and not attributable to either classical antisense mechanism or interferon response effectors PKR and RNaseL.

This study yielded insights into the accessibility of the viral genome to RNAi, and the ability of virus to escape this defense mechanism. Several siRNAs targeting regions in capsid and polymerase that are well conserved among enteroviruses were effective in inhibiting viral replication. In contrast, siRNA that targeted the 5′ non-coding region (5′UTR), also highly conserved among all picornaviruses, failed to reduce virus replication (L. Gitlin and R. Andino, unpublished observations). This result implies that some regions of the 5′UTR are less accessible to the RNAi machinery. Indeed, secondary and tertiary structure of the RNA, as well as proteins binding to RNA elements of the 5′UTR, could potentially shield the viral target RNA from the RNAi machinery. Since siRNAs directed against the hepatitis C virus IRES have been recently shown to effectively inhibit replication of HCV replicons (Yokota et al., 2003), there may be differences in the accessibility of different regulatory regions of different viral genomes. Notably, viruses can eventually become resistant to synthetic short RNAs such as those described by Gitlin et al. (2002). Escape mutants emerged from cells treated with single siRNAs after poliovirus infection at a high multiplicity of infection. These escape mutants presented subtle nucleotide changes in the siRNA target sequences. It appears that a single mismatch located approximately in the center of the siRNA nearly abolishes silencing of poliovirus (Gitlin and Andino, unpublished observations).

5. Therapeutic potential of RNAi as an antiviral agent

The susceptibility of viruses to RNAi in tissue culture suggests that RNAi-based antiviral strategies might be feasible also in entire mammals. Evidence that RNAi can be induced in vivo has been obtained in a number of mouse studies, in which injection of siRNA or plasmids encoding small hairpin RNAs (shRNAs) directed against luciferase, GFP, or a secreted form of human placental alkaline phosphatase (SEAP) could inhibit expression of these genes from plasmids after high pressure tail injection (hydrodynamic transfection) (Lewis et al., 2002; McCaffrey et al., 2002). Silencing of endogenous genes was shown by downregulation of GFP in a transgenic mouse (Lewis et al., 2002). The first application of RNAi technology to prevent disease was illustrated by protection of mice against antibody- or concanavalin A-induced hepatitis by using siRNA directed against *fas* protein (Song et al., 2003). Recently, shRNAs against HBV mRNAs were shown to be able to suppress virus replication from co-injected HBV expression plasmids in hepatocytes (McCaffrey et al., 2003), providing proof of principle for the use of RNAi-based antiviral strategies in animal models.

Despite these promising results, significant hurdles exist in the successful application of RNAi-based antiviral strategies in a clinical setting. Several of these issues may be addressed using poliovirus as a model virus.

First, as shown in Gitlin et al. (2002), a limited number of mismatches between siRNA and target RNA may already abolish the suppressive action of the siRNA (Elbashir et al., 2001; Martinez et al., 2002b). The error-prone nature of the virally encoded RNA-dependent RNA polymerase will generate a spectrum of mutants in each replication cycle, and it can be expected that escape mutants will rapidly arise under incomplete suppression by siRNA. Indeed, point mutations arose in the center of the target region of the poliovirus genome, when infections were performed at high MOI (Gitlin et al., 2002). Although single mismatches have been shown to render a siRNA ineffective (Elbashir et al., 2001), they may be tolerated by the RNAi machinery to some extent (Jacque et al., 2002). Simultaneously targeting of multiple regions or of regions that constitute indispensable RNA structures for virus replication and thus do not allow viral escape, might be approaches to circumvent viral escape, that can readily be explored using the poliovirus model system. Alternatively, targeting essential cellular factors, such as CC-chemokine receptor 5 (CCR5) and CD4 in the case of HIV-1 (Novina et al., 2002; Qin et al., 2003) or co-administration with other antiviral drugs may limit the possibility for viral escape.

A second aspect requiring further investigation is that not all siRNAs are equally effective in the suppression of a target RNA (Harborth et al., 2001). The sequence requirements for effective RNAi-based suppression have not been well defined and efficient siRNAs need to be developed by trial and error. It is likely that optimal siRNA sequences are determined by the interaction of the siRNAs with both multicomponent nuclease RISC and with the target RNA sequence. Other open questions that apply specifically to viral genomes as targets for RNAi, are whether there is a differential susceptibility to RNAi between coding and non-coding regions, and whether protein bound RNA

structures are less accessible to the RNAi machinery. If conserved secondary structures in the untranslated regions of the virus are indeed susceptible, these might be the regions of choice to develop siRNAs as mutations in these structures would lead to loss of function.

Another aspect that is not yet properly defined is whether it is possible to block viral replication once initiated. Effective inhibition of poliovirus was observed when cells were transfected with siRNA before infection (Gitlin et al., 2002). Although in theory siRNA could target newly synthesized RNA strands of either positive and negative orientation, replication of poliovirus on membranous vesicles and coupling of RNA synthesis with virion assembly might shield replicative intermediates from degradation by the RNAi machinery.

Virally encoded suppressors of the RNAi pathway have been described in a variety of plant viruses (Brigneti et al., 1998; Voinnet et al., 1999) and in FHV, which naturally infects vertebrate and invertebrate hosts (Li et al., 2002). Thus far, similar suppressors have not been reported in mammalian viruses. The presence of putative suppressors in specific mammalian virus families would limit the potential of RNAi-based therapeutic strategies.

Finally, recent RNAi studies in animals rely heavily on the efficient uptake and expression of siRNA in hepatocytes (Liu et al., 1999; Song et al., 2003; Zhang et al., 1999). Other methods to deliver shRNA by adenovirus-mediated delivery or lentiviral vectors have been successful in vivo as well (Tiscornia et al., 2003; Xia et al., 2002). However, as viruses differ greatly in tissue and cell tropism, specific targeting of siRNA expression systems to specific tissues will be crucial for the success of RNAi-based therapeutic approaches. These methods may therefore encounter similar problems and limitations as gene therapy (Thomas et al., 2003).

6. RNA silencing as a tool to study virus replication and pathogenesis

RNA interference has been developed into a powerful technique to manipulate gene expression experimentally, which may benefit the study of virus replication. For positive-stranded, nonsegmented viruses, such as poliovirus, any siRNAs directed against its genome would result in down regulation of the entire polyprotein. In contrast, for negative-stranded and segmented RNA viruses, such as paramyxoviruses and orthomyxoviruses, it is possible to downregulate specific viral genes using RNAi. This provides the basis for a novel approach in which individual viral proteins can be downregulated with RNAi to be replaced with modified proteins provided in *trans* (e.g., from plasmid vectors).

Viruses depend on host factors at every stage of their replication cycle. To establish the role of putative host factors in virus replication, RNAi-based downregulation may be a powerful approach. Traditional knockout strategies are labor intensive and may result in cellular toxicity, as can be expected for proteins that play important roles in basic biochemical processes in the host cell. For example, the poly A binding protein has been implicated in positive-stranded RNA virus replication, such as poliovirus (Herold and Andino, 2001; Svitkin et al., 2001). However, the protein is also involved in translation initiation and is essential for cell growth in *Saccharomyces cerevisiae* (Sachs et al., 1987). Transient, inducible and non-complete knock-down by RNAi may not interfere with cell survival, but still provide a window of opportunity to study the role of cellular factors in virus replication in the cell. Furthermore, complementation by an expression plasmid that encodes the protein of interest in a mutated form could provide additional information about sites of interaction.

For poliovirus, host factors involved in cap-independent translation initiation and negative strand RNA synthesis have begun to be defined through in vitro studies. Factors that have been implied in these processes include canonical translation initiation factors, poly A binding protein, poly(rC) binding proteins 1 and 2, La antigen, and poly-pyrimidine tract binding proteins (reviewed in Andino et al., 1999; Belsham and Sonenberg, 2000; Semler and Wimmer, 2002). Additional interactions with host factors include the interaction with the poliovirus receptor (CD155) for viral entry (Ren et al., 1990) and possible interactions with proteins of the COPII system leading to alterations in the secretory pathway to establish membranous vesicles which function as sites for virus replication (Rust et al., 2001). It is likely that in the near future approaches that employ downregulation of these factors using RNAi will yield insights in their specific function during viral replication.

7. Conclusion

Much information about the mechanism and the use of RNAi as a tool to manipulate gene expression has become available in recent years. A better understanding of this novel defense mechanism may result in effective therapeutic approaches for intractable viral diseases that are currently afflicting humans. However, although poliovirus (and other viruses) can be targeted by artificially introduced siRNA in vitro, still much research needs to be done to establish the role of RNAi in natural virus infection, and several critical issues need to be addressed, before RNAi-based technology might be used for the treatment of viral diseases.

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